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(57) Abstract

A technique is provided for linking an oligonucleotide, such as a single strand of DNA, to another moiety or solid phase. An oligonucleotide is provided with a linking functionality that can be defined by six neighboring hystaminyal purines, which can be coordinated to a metal ion that also is coordinated by a chelating linked to a solid phase or other moiety. The invention finds particular use in resolution of one strand of DNA from its complement by providing the first strand with the linking functionality and passing the DNA across a metal affinity chromatography solid phase.

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OLIGONUCLEOTIDE LINKER AND TECHNIQUES INVOLVING IMMOBILIZED AND LINKED OLIGONUCLEOTIDES

Field of the Invention

The present invention relates generally to the production of single-stranded DNA molecules, and more particularly to the production of single-stranded DNA using immobilized metal affinity chromatography.

Background of the Invention

Many of the most widely employed operations in molecular biology hinge upon the use of single-stranded DNA as a probe or template. Whereas single strands of DNA containing up to approximately 100 residues can readily be produced by solid-phase synthesis, longer oligodeoxynucleotides typically must be generated through enzymatic methods such as the polymerase chain reaction (PCR). Because biochemical procedures for the synthesis of mixed-sequence DNA yield double-stranded products, there exists a need for a method by which to resolve a desired strand from its complement. Such resolution is rendered difficult by the similarities in macroscopic physical properties such as size and charge of two complimentary strands, and by the requirement that resolution be carried out in the presence of strong denaturants such as urea or guanidinium hydrochloride.

Techniques exist for attaching a single biotin onto an oligonucleotide, then using a solid matrix at which is immobilized avidin or streptavidin, to immobilize the oligonucleotide. A problem with this approach is that the binding that is required for the technique to be effective depends upon proteins being properly folded. Therefore, the technique cannot be used under strong denaturing conditions. Also, if the technique is to be used to surface-immobilize a single DNA strand for surface plasmon resonance studies, the avidin/biotin combination adds enough mass to the surface that the technique, when used to detect binding of a single strand of DNA to the immobilized strand, can lack precision.

It is, therefore, a object of the present invention to provide improved techniques for linking an oligonucleotide such as DNA, or a single strand of DNA, to another moiety or a surface, and to resolve a desired strand of DNA from its complement.

Summary of the Invention

The present invention provides a nucleotide coupled to a linking moiety that can

coordinate a metal ion, namely, a species having a formula L_n-N_y, where N represents the nucleotide and L represents the moiety which can coordinate a metal ion. n is at least 1, and y is at least 1. According to one embodiment N_Y is DNA, and L can be histaminylpurine where n is approximately 6.

According to another aspect, the invention provides a species having a formula R-Ch-M-L_n-N_y. According to this aspect, R represents a chemical moiety that can be linked to a surface, or linked to a chemical or biological species. Ch represents a chelating agent that can coordinate a metal ion, M represents a metal ion coordinated by the chelating agent, N represents a nucleotide, L represents a moiety that can coordinate a metal ion, and n and y each is at least 1.

The chelating agent is, according to one embodiment, a quadradentate chelating agent, for example nitrilotriacetic acid.

The invention also provides a single stranded oligonucleotide immobilized on a surface and free of hybridization to any complimentary strand that is also immobilized on the surface to the extent that the complimentary strand cannot be removed under conditions that cause denaturization of oligonucleotide strand from its complimentary strand. The single-stranded immobilized oligonucleotide also will withstand conditions, while remaining immobilized, harsher than 2 molar urea. The strand can be a strand of DNA.

According to another aspect the invention provides a method. The method involves providing first and second complementary strands of oligonucleotide, contacting a surface of a solid phase with the first and second strands, and allowing the first strand to be immobilized at the surface. The second strand is allowed to be carried away from the surface without immobilization at the surface. According to one embodiment, this aspect involves providing first and second complementary strands of DNA in a fluid medium, passing the fluid medium across a surface of a solid phase, and allowing the first strand to be immobilized at the surface while the second strand is allowed to be carried by the fluid medium away from the surface without immobilization.

The various aspects of the invention can be combined in a variety of ways. For example, the single stranded oligonucleotide immobilized on a surface and free of hybridization to any complimentary strand that is also immobilized on the surface to the extent that the complimentary strand cannot be removed under conditions that cause denaturization, can be immobilized via linkages such as L_n-N_y or R-Ch-M-L_n-N_y, above. Additionally, the methods can be practiced using any of the species that can facilitate immobilization of an oligonucleotide.

Other advantages, novel features, and objects of the invention will become apparent from the following detailed description of the invention when considered in conjunction with the accompanying drawings.

Brief Description of the Drawings

Fig. 1 is a schematic representation of double-stranded DNA immobilized, via an attached linking moiety coordinated to a metal ion, to a solid phase surface;

Fig. 2 illustrates, schematically, primers and double-stranded PCR template used in one set of examples; and

Fig. 3 is a photocopy of a denaturing polyacrylamide gel electrophoresis analysis of DNA strand resolution using a technique of the present invention.

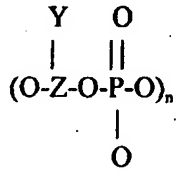
Detailed Description of the Invention

Min, C., Cushing, T. Verdine, G., "Template-Directed Interference Footprinting of Protein-Adenine Contacts,", *J. Am. Chem. Soc.* 1996, 118, 6116-6120; and Min, C. Verdine, G., "Immobilized Metal Affinity Chromatography of DNA," *Nucleic Acids Res.* 1996, 24, 3806-3810, both are incorporated herein by reference.

The present invention provides a linking functionality for attachment of an oligonucleotide to another moiety or to a surface of a solid phase, where solid phase is defined as any material insoluble in a medium used in conjunction with a procedure involving the oligonucleotide. The technique can be used, for example, to link an oligonucleotide to a solid phase surface such as a chromatography solid phase, or to a surface of a biosensor element such as a surface plasmon resonance (SPR) chip (for a discussion of SPR see, for example, Sternberg, et al., "Quantitative Determination of Surface Concentration of Protein with Surface Plasmon Resonance Using Radiolabeled Proteins", *Journal of Colloid and Interface Science*, 43:2 513-526, 1991, and references therein). The technique also can be used, for example, to link an oligonucleotide to a surface of a bead or plate used in a bioassay, to a label such as a fluorescent label, or another molecule or solid entity.

The invention involves providing a species having the generalized formula L_n-N_y, where N_y represents a natural or synthetic, single or multiple-strand oligonucleotide typified in DNA or a strand of DNA, and L_n represents a linking functionality where L represents a ligating moiety that can coordinate a metal ion, and n is at least 1, preferably at least 2, and more preferably at

least 5. According to preferred embodiments, n is from 2 to 10, and most preferably is about 6. L is a molecule that can be chemically attached to an oligonucleotide via, for example, the ester bond of the oligonucleotide backbone, and L_n has a generalized formula I, below.

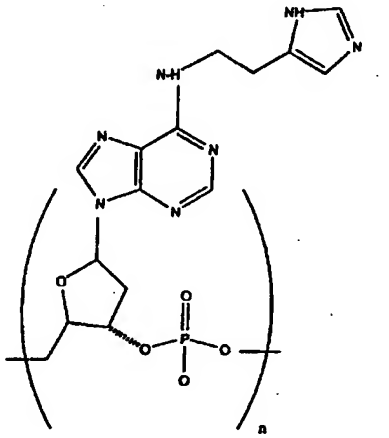


(I)

where Z is an organic moiety and Y is a ligand having the ability to coordinate a metal ion, Z and Y together allowing incorporation of L_n into or on an oligonucleotide sequence. Those of ordinary skill in the art can select appropriately Y and Z to meet the requirement of compatibility with an oligonucleotide sequence. Z and Y can be a single moiety in that a single organic moiety can provide functionality compatible with an oligonucleotide sequence and also coordinate a metal ion. Typically, Z is a hydrocarbon chain or a cyclic hydrocarbon group, optionally interrupted by hetero groups, of a length of from about 1 to about 10 carbon atoms. As used herein, "hydrocarbon" is meant to include alkyl, alkenyl, alkynyl, cycloalkyl, aryl, alkaryl, aralkyl, and the like. Hetero groups can include -O-, -CONH-, -CONHCO-, -NH-, -CSNH-, -CO-, -CS-, -S-, -SO-, $-(OCH_2CH_2)_nR$ (where $_n$=1-10), $-(CF_2)_n-$ (where $_n$=1-10), olefins, and the like.

Y (or Y and Z together) is a ligand that can coordinate free coordination sites on a metal ion, such as a metal ion immobilized on a metal affinity chromatography solid phase without complete coordination of the ion by the solid phase (see, for example, Hochuli et al., "New Metal Chelate Adsorbent Selective for Proteins and Peptides Containing Neighboring Histidine Residues" *Journal of Chromatography*, 411 (1987) 177-184). Y can be as defined above for Z, and preferably includes groups such as -N-, alcohols, thiols, carboxylates (including carboxylic acid), and the like which can address a vacant coordination site on a metal ion. Cyclic and heterocyclic organic compounds including -N- such as imidazoles, phenanthroline, adenine, cytosine, and thymine are suitable. Additionally, amino acids satisfying these criteria, such as histidine, lysine, cysteine, methionine, asparagine, tyrosine, glutamine, glutamate, and aspartate are suitable. Species such as hydroxamic acid also are suitable. In some cases, the above moieties can serve as Z, with a different one of the above or another moiety serving as Y and satisfying the criterion of coordination to a metal ion. For example, purine, thymine, adenine,

cytosine, or a combination could serve as Z, with another nucleic acid, amino acid, or nitrogen-containing cyclic, multi-cyclic, or heterocyclic compound serving as Y. Where, for example, purine, cytosine, or adenine are used as Z, the available $-NH_2-$ group can be used to couple Y defined by, for example, histamine. According to one preferred embodiment, 6-histaminyl purine defines Y, and Z is a cyclic ether (II, below).



(II)

Species L_n-N_y finds use in conjunction with a metal ion partially coordinated by a chelating agent that is linked, or can be linked, to a moiety or surface of interest. That is, the species finds use in a combination of the formula:

$$R\text{-}Ch\text{-}L_n\text{-}N_y \qquad \text{(III)}$$

where R represents a chemical or physical moiety, Ch represents a chelating agent that can coordinate a metal ion, and M represents a metal ion coordinated by the chelating agent. R can be a particulate species such as gold sol, a label such as a fluorescent label, a biological molecule of interest such as a binding partner for interaction with another species, a chromatography solid phase, or a linking or linkable moiety that can effect coupling of species III to any desired surface or species. The particular species or surface to which species III is coupled is not important, but the invention lies in the ability to coordinate N_y to R via L_n.

Co-pending, commonly-owned U.S. Patent Application Serial No. 08/312,388, filed September 26, 1994, now U.S. Patent No. 5,620,850, and incorporated herein by reference,

describes a variety of combinations of Ch and M suitable for use in the invention. In particular, the metal ion is preferably selected from those that have at least four coordination sites, preferably six coordination sites. A non-limiting list of metal ions suitable includes Co^{3+}, Cr^{3+}, Hg^{2+}, Pd^{2+}, Pt^{2+}, Pd^{4+}, Pt^{4+}, Rh^{3+}, Ir^{3+}, Ru^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Au^{3+}, Au^{+}, Ag^{+}, Cu^{+}, MO_2^{2+}, Tl^{3+}, Tl^{+}, Bi^{3+}, CH_3Hg^{+}, Al^{3+}, Ga^{3+}, Ce^{3+}, UO_2^{2+}, and La^{3+}.

The chelating agent is preferably selected from bidentate, tridentate, and quadradentate, chelating agents, and is selected in conjunction with the metal ion so that when the chelating agent coordinates the metal ion, at least two free coordination sites of the metal remain. The chelating agent and metal are selected so that the chelating agent can coordinate the metal ion with a degree of stability great enough that the metal ion will remain immobilized at the surface by the chelating agent.

Additionally, the chelating agent is selected as one that has a chelating moiety and a non-chelating linker moiety, such that it can be covalently linked via its linker moiety to the moiety R while leaving the chelating moiety undisturbed by the covalent linkage and free to coordinate a metal ion. Alternatively, the chelating agent can be selected as one that can be modified via routine organic synthesis to include a non-chelating linker moiety, if such synthesis leaves undisturbed the chelating moiety. One of ordinary skill in the art will appreciate that the non-chelating linker moiety should provide functionality suitable for chemical linkage such as, for example, an amine, alcohol, carbamate, carboxylic acid, thiol, aldehyde, olefin, etc., for formation of an ester linkage, formation of an amide linkage, thiol displacement and thiol ether formation, and the like.

With the above considerations in mind, suitable chelating agents and corresponding metal ions can be selected by those of ordinary skill in the art. In accordance with such selection reference can be made to "Chelating Agents and Metal Chelates", Dwyer, F. P.; Mellor, D. P., Academic Press, and "Critical Stability Constants", Martell, A. E.; Smith, R. M., Plenum Press, New York. These works describe a variety of chelating agents, and discuss the stability of coordination between chelating agents and metal ions. Preferably, a chelating agent and metal ion is selected such that the dissociation constant of the combination in aqueous solution is better than 10 nM at physiological pH, that is, such that at least one half of the metal ions are coordinated by chelating agent at a concentration of 10 nM.

A non-limiting exemplary list of suitable chelating agents includes nitrilotriacetic acid, 2,2'-bis(salicylideneamino)-6,6'-demethyldiphenyl, and 1,8-bis(a-pyridyl)-3,6-dithiaoctane.

As an example of one, preferred arrangement using species III, reference is made to Fig. 1, in which a species 25 linked to a solid phase surface includes an oligonucleotide, in particular DNA 10 including a first strand 12 and a second, complementary strand 14 including a linking functionality (L_n) 16 defined by 6 histaminyl purines, two of which (18 and 20, respectively) are coordinated to a metal ion 22. Metal ion 22 also is coordinated by chelating agent 24 (a nitrilotriacetic acid derivative, as illustrated) which coordinates four of the six coordination sites of the metal ion, leaving two free coordination sites addressable by linking functionality 16. Chelating agent 24 is attached, via linker 26 (a hydrocarbon chain, as illustrated), to a surface 28 of a solid phase 30. Linkage of chelating agent 24 to surface 28 via linker 26 can be accomplished by any means such as covalent attachment, or the like.

A single-stranded oligonucleotide also is usefully immobilized as shown in FIG. 1 in accordance with the invention. According to one embodiment, linker 26 is selected as one that promotes formation of a self-assembled monolayer of a plurality of linkers 26 at surface 28, and linker 26 terminates at its end opposite the chelating agent in a functionality that is attracted to surface 28 and thereby promotes formation of a self-assembled monolayer. Such an arrangement of a metal-coordinating chelating agent immobilized at a surface as part of a self-assembled monolayer is described in commonly-owned co-pending U.S. patent application serial number 08/312,388, now U.S. Patent No. 5,620,850, referenced above. Self-assembled monolayer-forming species also are described in U.S. Patent No. 5,512,131, issued April 30, 1996 to Kumar and Whitesides, incorporated herein by reference. Where a self-assembled monolayer containing a chelating agent 24 is provided for use with species L_n-N_y such as DNA 10 including linking functionality 16, it can be advantageous to form a self-assembled mixed monolayer (as defined in application serial number 08/312,388, and U.S. Patent No. 5,620,850 referenced above), that is, a heterogeneous self-assembled monolayer including an ordered assembly of at least another self-assembled monolayer-forming species other than species 25. A heterogeneous monolayer defined by less than about 50% of species 25 and greater than about 50% of another species such as one terminating in a moiety that does not interact with L_n or N_y can be formulated, as well as one with a ratio of less than about 30% species 25, or less than about 20% species 25, etc.

It is to be understood that coordination of linking functionality 16 to M may involve linkage via two neighboring ligating moieties L (H, for histaminyl purine, as illustrated in FIG. 1), or another arrangement. Additionally, linking functionality 16 may include neighboring ligating moieties L and/or ligating moieties L separated by one or more intermediate moieties

that do not have ligating capacity, so long as linking functionality 16 possesses the ability to coordinate free coordination sites at M.

Referring again to formula I, Y (and Y in combination with Z if Z has coordinating capacity) and n should be selected to provide the oligonucleotide with coordinating capacity sufficient to link the oligonucleotide to a metal ion to the extent that the oligonucleotide does not become detached from the metal ion during the course of a procedure involving the oligonucleotide coordinated to the metal ion so as, for example, to immobilize the oligonucleotide at a solid phase. Coordination should not, however, be so strong that L_n will compete with coordination of the metal ion by the chelating agent to the extent that the metal ion is stripped from the chelating agent (thus, e.g., stripped from a solid phase, label, or other moiety). For example, in most cases ligands such as ethylenediamine tetra acetic acid would be unsuitable. Those of ordinary skill in the art, with reference to "Chelating Agents and Metal Chelates," Dwyer, F.P.; Mellor, D.P., Academic Press, and "Critical Stability Constants", Martell, A.E.; Smith, R.M., Plenum Press, New York, can select a suitable group Y (and optionally Z) and the number of repeating units n for a particular metal ion including a particular number of free coordination sites.

In some cases it can be advantageous to test a particular L_n/M-Ch system to determine whether coordination will be sufficiently stable for use in the present invention, but will not be so stable that the metal ion is stripped from the chelating agent. For example, it is within the realm of routine experimentation to one of ordinary skill in the art to prepare a very simple polynucleotide containing as few as two nucleotides or up to approximately 6 or 8 with an attached linking functionality L_n, i.e., 3'-NNNNN-L_6, where N is any nucleotide or a variety of nucleotides. The test oligonucleotide then can be labeled radioactively at the 5' end by mixing with T_4 polynucleotide kinase, then with γ^{32}P-ATP. Then the oligonucleotide can be mixed with beads of resin containing immobilized, coordinated metal ion, the beads rinsed, and determination can be made as to whether radioactivity is present at the beads. If radioactivity is present, then the selection of L (i.e. Y or Y and R together) and n is appropriate to provide coordination capacity sufficient to immobilize the oligonucleotide, but not to the extent that the metal ion is stripped from the chelating agent.

If radioactivity is not present at the beads, then determination of whether the L, n combination results in insufficient coordination or coordination to an excessive extent such that metal ion is stripped can be made by subjecting the beads, and/or the solution used to flush the

beads, to ultraviolet spectroscopy or ICP to determine the location of the metal ion. These simple screening techniques can be performed by one of ordinary skill in the art without the necessity of synthesizing an entire oligonucleotide such as a DNA fragment useful in a particular procedure.

In some cases one would want to be able to separate the linker-nucleotide from the metal ion, as in elution from a column, and in some cases one would want essentially irreversible binding. Accordingly, as a second test, beads can be flushed with an eluting fluid such as 200mM imidazole and determination made as to whether the radioactivity is still present at the beads.

The present invention also represents an improvement over immobilization of single-stranded oligonucleotide at a surface via biotin/avidin or biotin/streptavidin linkage. That linkage will withstand conditions only up to 2 molar urea, or less. The present invention involves immobilization that withstands conditions harsher than 2 molar urea.

The function and advantage of these and other embodiments of the present invention will be more fully understood from the examples below. The following examples are intended to illustrate the benefits of the present invention, but do not exemplify the full scope of the invention.

Example 1: Synthesis Of A Nucleotide Including A Moiety That Can Coordinate A Metal Ion

A nucleotide that can coordinate a metal ion due to incorporation of a moiety for that purpose (having a formula L_n-N_y), specifically, DNA sequencing primers containing six successive 6-histaminylpurine residues added on to the 5'-end of the DNA was synthesized. The 6-histaminylpurine moiety was introduced through the convertible nucleotide approach by analogy to reported procedures (Ferentz, A.E.; Verdine, G.L., *J. Am. Chem. Soc.* 1991, *113*, 4000-4002; Ferentz, A.E.; Verdine, G.L. In *Nucleic Acids and Molecular Biology*; Eckstein, F., Lilley, D.M.J. Eds.; Springer-Verlag: Berlin, 1994; Vol. 8, pp. 14-40. Briefly, resin-bound oligonucleotides containing O^6-phenyl-2'-deoxyinosine (ϕdI) in place of the dH residue were deprotected from the resin by mild ammonia treatment (conc NH_4OH, nt, 4b) lyophilized to dryness, then treated with 5 M aqueous histamine (55°C, 14 h) to convert the ϕdI residues to dH. The crude oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis. An additional dG residue was added at the 5'-end to ensure efficient end-labeling using polynucleotide kinase.

For the present IMAC-based strategy to be of significant practical value, it must be capable of resolving PCR-amplified duplex DNA into its two component strands. To test this application directly, we used one H_6-tagged primer plus one unmodified primer to amplify a 183 base-pair segment of the plasmid pUC18-mARRE2, thereby to furnish a duplex product with an H_6-tag on only one strand. The DNA template for PCR amplification, pUC18-mARRE2, was derived from the commercial cloning vector pUC18 by inserting a segment of the murine interleukin-2 enhancer into the *BamH* I site (Chen, L.; Jain, J.; Oakley, M.G.; glover, J.N.M.: Dervan, P.B.: Hogan, P.G.: Rao, A.; Verdine, G.L. *Curr. Biol.* 1995, 5, 882-889). pUC18 and close relatives are more widely used in bacterial cloning than each of the unbound and bound fractions was added 900 µl absolute ethanol (stored at 20°C), then the tubes were vortexed briefly and chilled for 30 min on dry powdered CO_2. The tubes were microcentrifuged for 30 min at 16,000 x g. The supernatant was removed and the pellet washed with 200 µl 80% aqueous EtOH (-20°C). Following removal of the ethanol solution, the tubes were dried by centrifugal lyophilization (SpeedVac, Savant). To each dry tube was added 50 µl TE buffer. The DNA concentration was determined by UV spectrophotometry. The designation of "top" and "bottom" strands refers to the sequence as mapped in Figure 2. The "top" strand also corresponds to the coding strand of the lacZ'α peptide, part of which was encoded within the polylinker region of pUC18. That is, the PCR product generated using primers 1a + 2b will bear the H_6-tag on the "top" strand only, whereas that generated using 2a + 1b will bear the H_6-tag on the "bottom" strand only. The yield of PCR product formed in these reactions was no less than that observed in parallel reactions with only unmodified primers (data not shown), thus indicating that the H_6-tag does not affect PCR amplification adversely.

Primers 1a/1b contain a sequence that is identical to a stretch (shaded) of the "top" strand of the plasmic pUC18-mARRE2; they are extended in the rightward direction (5'-3') during PCR. Primers 2a/2b contain a sequence that is identical to a stretch (shaded) of the "bottom" strand of pUC18-mARRE2; they are extended in the leftward direction. The shaded sequence in primers 1a and 2a denotes the H_6-tag. *E* and *H* denote the *Eco*R I and *Hnd* III sites, respectively, of the pUC18 polylinker; a stretch of the murine IL-2 enhancer (mARRE2, batched) was inserted to the *Bam*H I site to generate pUC18-mARRE2. (c) Schematic illustration of the procedure for PCR amplification to produce duplex DNA containing a H_6-tag on one strand, and resolution of the two constituent strands using IMAC.

Example 2: Immobilization Of L_n-N_y At A Surface

The nucleotide including a moiety that can coordinate a metal ion was immobilized at a surface. Specifically, to assess the ability of the H_6-tag to endow DNA with selective retentivity on a Ni^{2+}-charged chelating resin, the H_6-tagged oligonucleotides (2a/2b) and their unmodified counterparts (1b/2b) were 5'-end-labeled with ^{32}P, then passed in parallel through a Ni^{+2}-nitrilotriactic acid (NTA)-agarose resin. In particular, the PCR products were denatured in 6- M guanidinum·HCl, then incubated batch wise with Ni_{2+}-NTA-agarose.

Example 3: Immobilization of a First Strand of First and Second Complementary Strands of DNA at a Surface with Selective Removal of the Second Strand, and Quantitative Determination of Bound Versus Unbound Strands

Following the procedure of Example 2, it was demonstrated that the H_6-tag confers, upon an oligonucleotide, the ability to be selectively and reversibly retained on a surface, specifically Ni^{2+}-NTA-agarose. In particular, it was demonstrated that the technique results in single-stranded oligonucleotide immobilized on a surface and free of hybridization to any complimentary strand that is also immobilized on the surface to the extent that the complimentary strand cannot be removed under conditions that cause denaturization of oligonucleotide strand from its complimentary strand.

Following removal of the supernatant, which contains unbound DNA, the resin was washed and the bound DNA eluted with 200 mM imidazole. Aliquots of the crude PCR product and the two DNA-containing fractions from the IMAC step were 5'-end labeled with ^{32}P and analyzed by denaturing polyacrylamide gel electrophoresis (PAGE). The use of PAGE as an assay for strand resolution was made possible by the slightly reduced mobility of H_6-tagged DNA strands relative to their complimentary strands generated by PCT. The retarded mobility of the H_6-tagged strand relative to the unmodified strand may arise from the partial positive charge of its 6 pendant imidazole moieties at pH 8 and a greater length, due to incomplete polymerization of the T_6-stretch opposite H_6 during PCR. The identities of the strands were independently verified by PAGE analysis of PCR reactions that employed one ^{32}P-labeled primer and one nonradioactive primer. Although this slight difference in mobility provides a convenient means of assaying the strand resolution by IMAC, it is not sufficiently large to permit preparative strand resolution by gel electrophoresis and extraction.

Fig. 3 is a photocopy of the results of denaturing PAGE of DNA strand resolution using IMAC. Each panel of three lanes represents the results obtained using a pair of PCR primers,

one of which (1a or 2a) contains an H6-tag and the other of which (2b or 1b, respectively) is unmodified. C (lanes 1 and 4): controls showing the mixture of strands obtained directly by PCR, prior to IMAC resolution; U (lanes 2 and 5): unbound fractions from IMAC; B (lanes 3 and 6): bound fraction from IMAC, after elution with 200 mM imidazole.

The unbound fractions from IMAC in Fig. 3 contained primarily the faster-migrating species, which corresponds to the respective unmodified strands, whereas the imidazole-eluted bound fractions contained primarily the respective H_6-tagged strands. Quantitative phosporoimaging analysis from repeated runs of the strand resolution procedure revealed that the unmodified strand typically comprise >90% of the unbound DNA and the H_6-tagged DNA comprise 95% of the bound fractions. Thus we conclude that IMAC cleanly resolves a uniquely H_6-tagged PCR product into its two component strands. All but a trace amount of the bound radioactivity could be eluted from the resin upon washing with a 200mM aqueous imidazole.

Example 4: Template-Directed Polymerization Using Surface-Immobilized N_n-N_y

To assay the biological activity of single-stranded DNA generated through the PCR-IMAC sequence, we employed the resolved strands as templates in Sanger dideoxy sequencing runs. The experiment was carried out using oligonucleotide strands that had been synthesized so as to be attachable to a surface, in accordance with the invention, but were not attached to the surface during the experiment. Thus, the experiment proves the concept that the single-stranded oligonucleotide, immobilized at a surface in accordance with the invention, retains its biological activity.

Specifically, single-stranded DNA was immobilized at a surface of a column, as described above, and then the surface-immobilized DNA was flushed from the column, and a chelating agent was used to complex any remaining or free nickel ion in solution. Initially, the unmodified strands were found to be excellent sequencing templates, but the H_6-tagged strands failed to support template-directed polymerization. Eventually, it was discovered that the H_6-tagged DNA solutions contain adventitious Ni^{2+} in amounts sufficient to cause profound inhibition of the DNA polymerase enzyme. This problem was overcome simply by adding 1,10-phenanthroline to the imidazole-containing eluate prior to precipitation with ethanol. Single-stranded templates prepared in this manner give uniformly high-quality DNA sequence data.

Those skilled in the art would readily appreciate that all parameters listed herein are meant to be exemplary and actual parameters will depend upon the specific application for which

the methods and arrangements of the present invention are being used. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and at that, within the scope of the appended claims and equivalence thereto, the invention may be practiced otherwise and as specifically described.

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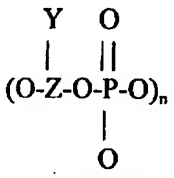
What is claimed is:

CLAIMS

1. A species having a formula L_n-N_y, where N represents a nucleotide, L represents a moiety that can coordinate a metal ion, n is at least one, and y is least one.

2. A species as in claim 1, wherein L_n has a generalized formula:



where Z is an organic moiety and Y is a ligand having the ability to coordinate a metal ion, Z and Y together allowing incorporation of L_n into or on an oligonucleotide sequence.

3. A species as in claim 2, wherein Z is a hydrocarbon chain or a cyclic hydrocarbon group, optionally interrupted by hetero groups, of a length of from about 1 to about 10 carbon atoms.

4. A species as in either of claims 2 or 3, wherein Y, or Y and Z together, is a ligand that can coordinate free coordination sites on a metal ion, such as a metal ion immobilized on a metal affinity chromatography solid phase without complete coordination of the ion by the solid phase.

5. A species as in any of claims 2-4 wherein, wherein Y includes -N-, alcohols, thiols, carboxylates, imidazoles, phenanthroline, adenine, cytosine, thymine, histidine, lysine, cysteine, methionine, asparagine, tyrosine, glutamine, glutamate, aspartate and hydroxamic acid, or a combination, that can address a vacant coordination site on a metal ion.

6. A species as in any preceding claim, wherein L represents a moiety that can coordinate a metal ion immobilized at a chromatography solid phase surface.

7. A species as in any preceding claim, wherein L is histaminylpurine and n is six.

8. A species as in any preceding claim, wherein N_y is DNA.

9. A species having a formula R-Ch-M-L_n-N_y, wherein R represents a chemical or

physical moiety, Ch represents a chelating agent that can coordinate a metal ion, M represents a metal ion coordinated by the chelating agent, N represents a nucleotide, L represents a moiety can coordinate a metal ion, and n and y each is at least one.

10. A species as in claim 9, wherein R is a gold sol, a label such as a fluorescent label, a biological molecule of interest such as a binding partner for interaction with another species, a chromatography solid phase, or a linking or linkable moiety that can effect coupling of the species to any desired surface or species.

11. A species as in either of claims 9 or 10, wherein R is a species that promotes formation of a self-assembled monolayer of a plurality of similar species.

12. A species as in any of claims 9-11, wherein Ch and M are together selected such that the dissociation constant of the combination in aqueous solution is better than 10 nM at physiological pH.

13. A species as in any of claims 9-12, wherein Ch is nitrilotriacetic acid, 2,2'-bis (salicylideneamino)-6, 6'-demethyldiphenyl, or 1,8-bis (a-pyridyl)-3, 6-dithiaoctane.

14. A species as in any of claims 9-13, wherein Ch is a quadradentate chelating agent.

15. A species as in claim 14, wherein Ch is nitrilotriacetic acid.

16. A species as in any of claims 9-15, wherein Ch and M are together selected such that the chelating agent coordinates all but at least two of the metal coordination sites.

17. A species as in any of claims 9-16, wherein Ch is a tetradentate chelating agent, and M has a coordination number of six.

18. A species as in any of claims 9-17, wherein M is Ni^{2+}.

19. A method comprising:

providing first and second complementary strands of oligonucleotide;

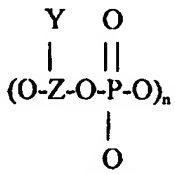
contacting a surface of a solid phase with the first and second strands; and

allowing the first strand to be immobilized at the surface while allowing the second strand to be carried away from the surface without immobilization at the surface wherein the first strand is immobilized at the surface via a linkage able to withstand conditions harsher than 2 molar urea.

20. A method as in claim 19, wherein the providing step comprises providing first and second complementary strands of DNA in a fluid medium, and the contacting step comprises passing the fluid medium across a surface of a solid phase.

21. A method as in either of claims 20-21, wherein the providing step involves providing first and second complementary strands of oligonucleotide, the first strand represented by N_y where N is a nucleotide and the oligonucleotide is part of a species having a general formula L_n-N_y, where L represents a moiety that can coordinate a metal ion, n is at least one, and y is least two.

22. A method as in claim 21, wherein L_n has a generalized formula:



where Z is an organic moiety and Y is a ligand having the ability to coordinate a metal ion, Z and Y together allowing incorporation of L_n into or on an oligonucleotide sequence.

23. A method as in claim 22, wherein Z is a hydrocarbon chain or a cyclic hydrocarbon group, optionally interrupted by hetero groups, of a length of from about 1 to about 10 carbon atoms.

24. A method as in either of claims 22 or 23, wherein Y, or Y and Z together, is a ligand that can coordinate free coordination sites on a metal ion, such as a metal ion immobilized on a metal affinity chromatography solid phase without complete coordination of the ion by the solid phase.

25. A method as in any of claims 22-24 wherein, wherein Y includes -N-, alcohols, thiols, carboxylates, imidazoles, phenanthroline, adenine, cytosine, thymine, histidine, lysine, cysteine, methionine, asparagine, tyrosine, glutamine, glutamate, aspartate and hydroxamic acid, or a combination, that can address a vacant coordination site on a metal ion.

26. A method as in any of claims 21-25, wherein L represents a moiety that can coordinate a metal ion immobilized at a chromatography solid phase surface.

27. A method as in any of claims 21-26, wherein L is histaminylpurine and n is six.

28. A method as in any of claims 21-27, wherein N_y is DNA.

29. A method as in either of claims 19 or 20, the allowing step involving allowing the first strand to be immobilized at the surface via a linkage R-Ch-M-L_n-N_y, wherein R represents the surface, Ch represents a chelating agent that can coordinate a metal ion, M represents a metal ion coordinated by the chelating agent, N_y represents the first strand, L represents a moiety can coordinate a metal ion, n is at least one, and y is at least two.

30. A method as in claim 29, wherein R is a surface of gold sol or a surface of a chromatography solid phase.

31. A method as in claim 29, wherein R is a surface of a chromatography solid phase.

32. A method as in any of claims 29-31, wherein Ch and M are together selected such that the dissociation constant of the combination in aqueous solution is better than 10 nM at physiological pH.

33. A method as in any of claims 29-32, wherein Ch is nitrilotriacetic acid, 2,2'-bis (salicylideneamino)-6, 6'-demethyldiphenyl, or 1,8-bis (a-pyridyl)-3, 6-dithiaoctane.

34. A method as in any of claims 29-33, wherein Ch is a quadradentate chelating agent.

35. A method as in claim 34, wherein Ch is nitrilotriacetic acid.

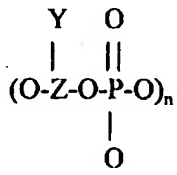
36. A method as in any of claims 29-35, wherein Ch and M are together selected such that the chelating agent coordinates all but at least two of the metal coordination sites.

37. A method as in any of claims 29-36, wherein Ch is a tetradentate chelating agent, and M has a coordination number of six.

38. A method as in any of claims 29-37, wherein M is Ni^{2+}.

39. A species comprising a single-stranded oligonucleotide immobilized on a surface and free of hybridization to any complimentary strand that is also immobilized on the surface to the extent that the complimentary strand cannot be removed under conditions that cause denaturization of oligonucleotide strand from its complimentary strand, the single-stranded oligonucleotide being immobilized via linkage able to withstand conditions harsher than 2 molar urea.

40. A species as in claim 39, wherein the single-stranded oligonucleotide is immobilized via incorporation into a species having a formula L_n-N_y, where N_y represents the oligonucleotide, L represents a moiety that can coordinate a metal ion, n is at least one, and y is least one, L_n has a generalized formula:



where Z is an organic moiety and Y is a ligand having the ability to coordinate a metal ion, Z and Y together allowing incorporation of L_n into or on an oligonucleotide sequence.

41. A species as in claim 40, wherein L is histaminylpurine and n is six.

42. A species as in any of claims 39-41, wherein the single-stranded oligonucleotide is single-stranded DNA.

43. A species as in any of claims 39-42, wherein the single-stranded oligonucleotide is part of a species having a formula R-Ch-M-L_n-N_y, wherein R represents a chemical or physical moiety, Ch represents a chelating agent that can coordinate a metal ion, M represents a metal ion coordinated by the chelating agent, N_y represents the single-stranded oligonucleotide, L represents a moiety can coordinate a metal ion, and n is at least 2, and y is at least one.

44. A species as in claim 43, wherein R is a chromatography solid phase.

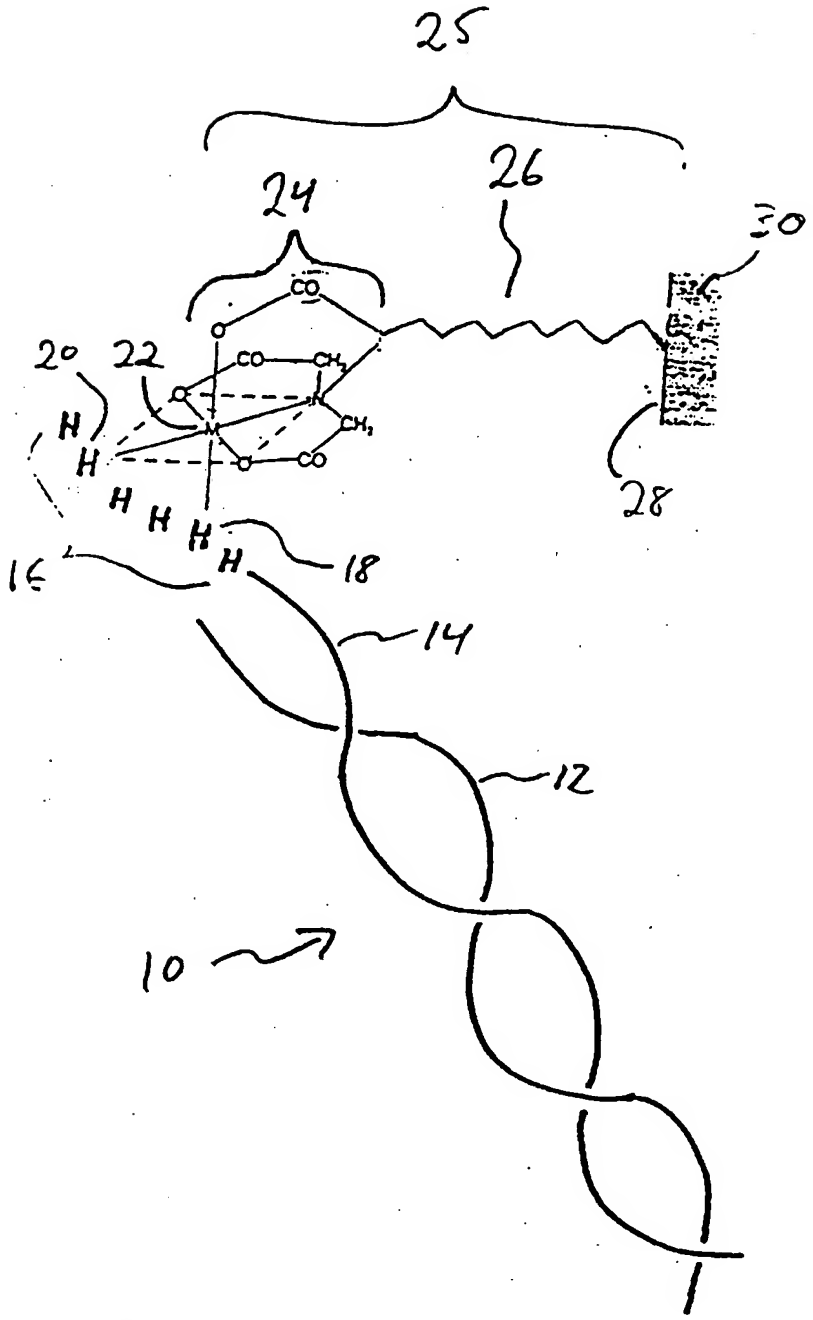


FIG. 1

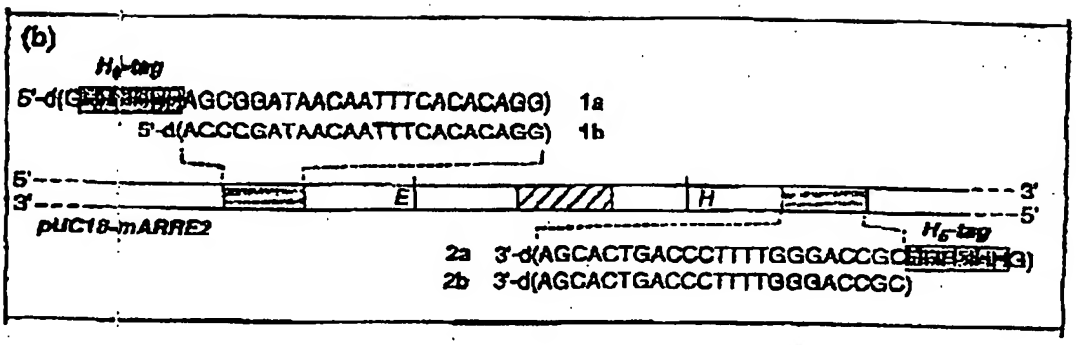

(b)
H_6-tag
5'-d(G[illegible]AGCGGATAACAATTTCACACAGG) 1a
5'-d(ACCCGATAACAATTTCACACAGG) 1b
5'
3'
E
H
3'
5'
pUC18-mARRE2
H_6-tag
2a 3'-d(AGCACTGACCCTTTTGGGACCGC[illegible]H3)
2b 3'-d(AGCACTGACCCTTTTGGGACCGC)

FIG. 2

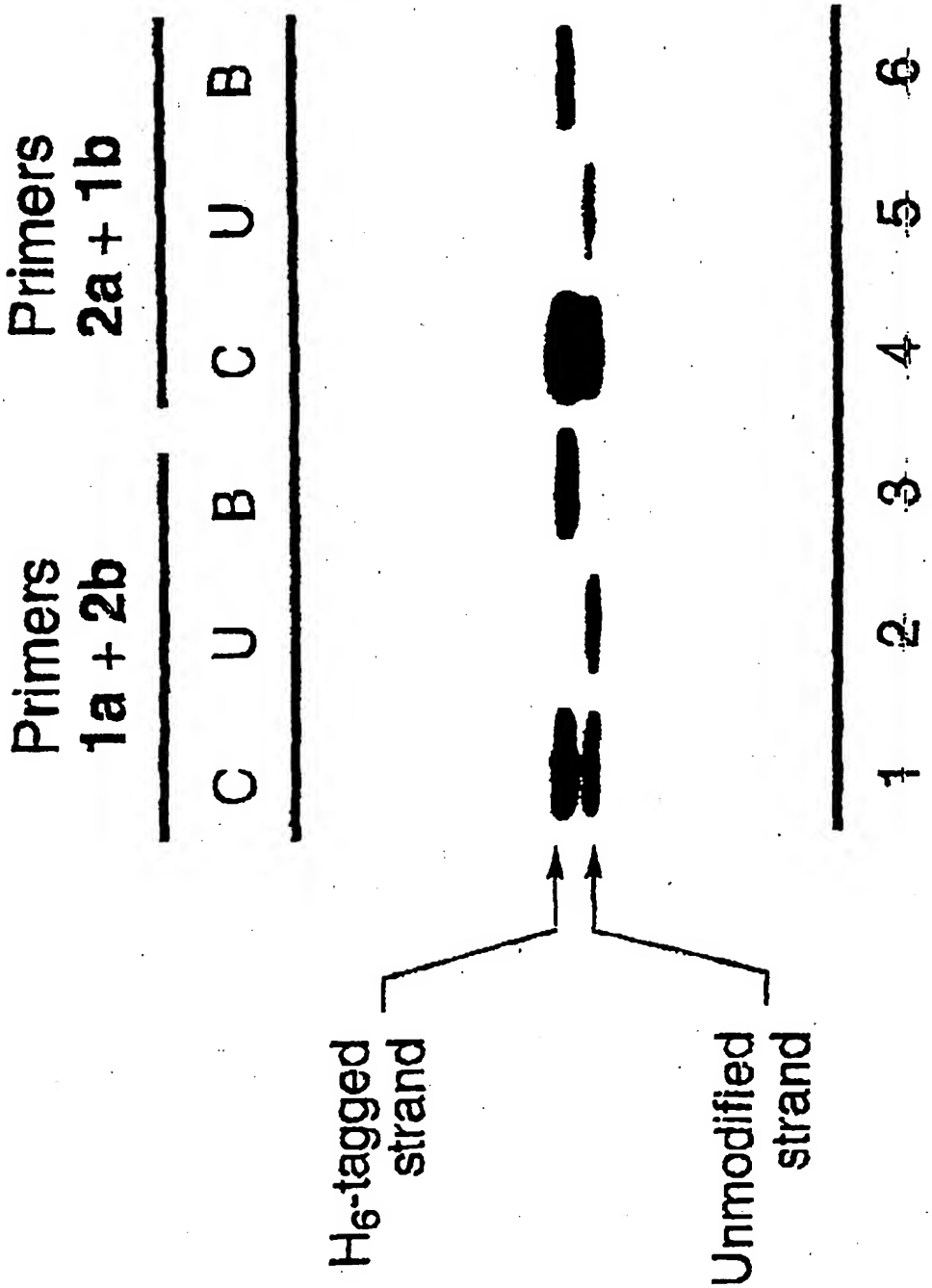

Primers
1a + 2b
C U B
Primers
2a + 1b
C U B
1 2 3 4 5 6
H_6-tagged strand
Unmodified strand

FIG. 3